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(54) Title: HUMAN MONOCLONAL ANTIBODIES AGAINST RABIES VIRUS

(57) Abstract

A human monoclonal antibody which: (a) binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae, and (b) neutralises said viruses; is useful for treating rabies in an infected individual and also for assaying for such a rabies virus.

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HUMAN MONOCLONAL ANTIBODIES AGAINST RABIES VIRUS

This invention concerns human monoclonal antibodies, hybrid cell lines that produce the antibodies and the use of the monoclonal antibodies.

Viruses of the rabies group of Rhabdoviridae comprise viruses that are infectious for nervous tissue of all warm-blooded animals. Rabies is usually contracted from the bite of an infected animal, but may also occur as a result of scratches or abrasions caused by an object contaminated with infected saliva, or rarely by penetration of mucous membranes, by exposure to a virus aerosol or the transplantation of infected tissues. See Harrison's Principles of Internal Medicine, 10th Edition, Petersdorf et al., eds., McGraw-Hill Book Company, p. 1136 (1983).

The incidence of the disease in unvaccinated individuals bitten by rabid animals is about 15 percent and varies depending on the amount of the virus in the saliva and the location and depth of the wounds. After an incubation period, usually 30 to 70 days, rabies gives rise to an invariably fatal disease in man. The first, or excitement, phase of the disease is characterised by fever, increasing agitation, spasmodic gross muscle contractions and generalised clonic or tonic convulsions with opisthotonos often precipitated by loud noises, bright lights or touch. The most typical feature of the disease is severe, painful contractions of the pharyngeal muscles, initially precipitated by attempts to swallow fluids which leads to hydrophobia (fear of water). To avoid swallowing, patients allow saliva to drool from the mouth. Death usually follows a generalised convulsion with prolonged apnea.

Patients who survive the excitement stage of the

aspect, this invention is directed toward the production of such human anti-rabies antibodies.

Kohler, G., and Milstein, C., Nature 256:496 (1975), were the first to exploit somatic cell hybridisation to make immortal hybridomas that continuously secrete monoclonal antibodies. In their work, they used plasmacytomas and lymphocytes of murine origin. Mouse antibodies produced in this fashion have proved useful for research and diagnostic purposes and some have even been used therapeutically in humans. Since the pioneering work of Kohler and Milstein, however, it became clear that human antibodies of similar specificity and reproducibility would represent a considerable advance in human immunoglobulin therapy. In 1980, two groups of investigators reported independently the application of Kohler and Milstein's technique to human cells [Croce, C.M., et al., Nature 288:488-489 (1980); Olsson, L., and Kaplan, H.S., Proc. Natl. Acad. Sci. USA 77:5429-5431 (1980)]. Since then, the production of human monoclonal antibodies against several antigens has been reported in the literature, although the total number described so far is much lower than the number of murine monoclonal antibodies reported.

Several approaches to the problem of producing human monoclonal antibodies have been developed, which include:

(I) Transformation of normal human lymphocytes with Epstein-Barr virus (EBV).

(II) Fusion of human lymphocytes to mouse myeloma cells.

(III) Fusion of human lymphocytes to mouse/human heteromyeloma cells.

(IV) Fusion of human lymphocytes to human myeloma or lymphoblastoid cells.

(V) A combination of procedure (I) with any one of procedures (II), (III), or (IV).

Figure 2 shows the detection of neutralizing anti-rabies virus antibodies in supernatants of clones derived from hybridoma 208. A to F represent controls as above. Supernatants of the clones were tested at a 1:10 final dilution. Three panels were tested:
a: neutralization was evaluated upon incubation of supernatants with 10^5 infectious rabies virus particles
b: with 10^3 infectious rabies virus particles; and
c: with 10^2 infectious rabies virus particles.

Figure 3 shows immunofluorescence studies with antibodies produced by clone 208/97 (1, 3) or with a polyclonal anti-nucleocapside antiserum (2, 4) on normal cells (3, 4) or cells infected with rabies virus (1, 2). Immunofluorescence tests were carried out on fresh (A) or fixed (B) cells.

Figure 4 shows the neutralizing potency of the monoclonal anti-rabies antibodies produced by clone 208/97 and of a reference equine anti-rabies serum. Δ: antibodies produced by clone 208/97 (10 µg/ml final concentration); ■ : reference antiserum 0.5 I.U./ml; O: reference antiserum, 0.05 I.U./ml; ✱ : reference antiserum, 0.005 I.U./ml.

Figure 5 shows the antiviral activity of the monoclonal antibodies produced by clone 208/97 and of a reference antiserum on rabies virus particles adsorbed to target cells. ■ : monoclonal antibodies produced by clone 208/97; □ : reference equine anti-rabies serum.

As used herein, the term "rabies virus" means viruses of the serotype 1 rabies group of Rhabdoviridae as defined in the WHO Technical Report Series, No. 709 (1984), and comprises both "street rabies viruses", as used to designate the agents of the naturally occurring disease, as well as "fixed rabies viruses", as used to designate rapidly multiplying strains used in vaccine production which have lost their infectivity for salivary gland tissue

after passage in the laboratory.

With respect to characterising the claimed hybrid cell lines, the terms "permanent" and "stable" mean viable over a prolonged time, typically at least about six months. The invention enables stable, permanent hybridoma cell lines to be provided which maintain the ability to produce the specified monoclonal antibody through at least 25 passages.

The term "monoclonal antibody" refers to an antibody selected from antibodies whose population is substantially homogeneous, i.e. the individuals of the antibody population are identical except for naturally occurring mutations.

The term "antibody" is also meant to include intact molecules as well as fragments thereof, such as Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of antibody, clear more rapidly from the circulation and may have less non-specific tissue binding than intact antibody. It will be appreciated that Fab, $F(ab')_2$ and other fragments of the monoclonal antibody of the present invention may be used as well as the intact antibody for the detection and treatment of rabies viral infection according to the methods of the present invention.

The term "neutralise" is used to denote the ability of antibody-containing supernatants to block the capacity of infectious rabies virus to infect target cells.

The human monoclonal antibodies of the invention:

(a) bind specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae, and

(b) neutralise said viruses.

Such a monoclonal antibody is secreted by a hybridoma cell line which has been prepared using cells of an immortalizing cell line and cells derived from a human which produce antibody having the specificity (a) above.

The immortalizing cell line is a cell line which, for practical purposes, can be maintain d perpetually in cell culture. In other words, it is stable and permanent and, when fused with cells which do not exhibit these properties, is able to confer the properties on the fusion product.

Any appropriate immortalizing cell line may be used. Typically, a plasmacytoma (myeloma) or lymphoblastoid cell line of mammalian origin may be employed such as a lymphoblastoid cell line of human origin. A preferred type of cell line is a human hypoxanthine-phosphoribosyl-transferase (HPRT) deficient and ouabain-resistant lymphoblastoid cell line. One such cell line is particularly preferred. This is the cell line FD5 which has been deposited at the European Collection of Animal Cell Cultures (ECACC), Porton Down, GB on 19 February 1988 under accession number 87061701.

This HPRT-deficient and ouabain-resistant mutant cell line was derived from the human B-lymphoblastoid cell line GM 1500A (obtained from the Human Cell Depository, Camden, New Jersey, U.S.A.). An HPRT-deficient mutant was obtained through selection in the presence of 20 μg/ml 8-azaguanin of an ethyl-methane sulphonate mutagenized cell population. In order to render the mutant thus-obtained ouabain resistant, the HPRT-deficient line was cultured in medium containing increasing concentrations of ouabain (up to 10^{-5}M). FD5 cells produce their own low amounts of IgG antibodies (~ 75 ng/10^6 cells/day). They grow in suspension as a typical lymphoblastoid line, forming large cell clumps. The growth medium consists of RPMI 1640 (Flow) supplemented with 20% heat inactivated foetal calf serum. The doubling time is 24 hours.

The immortalizing cells are fused with cells derived from a human which produce antibody which binds specifically to viruses of the serotype 1 group of the

rabies group Rhabdoviridae. These antibody-producing cells are lymphocytes which may or may not have been transformed with a virus. Preferably, Epstein-Barr virus (EBV) is used for transformation. Typically, it is the B-lymphocytes which are separated and fused with the immortalizing cells.

The antibody-producing cells are generally obtained from a human volunteer immunised with respect to a rabies virus of serotype group 1. A commercially available rabies virus vaccine may be used for this purpose. Peripheral blood lymphocytes (PBL) are obtained from the volunteer. The B-lymphocytes or at least a B-cell rich fraction of the PBL is generally obtained and transformed with a virus if desired. The lymphocytes may be restimulated in vitro with a rabies virus of the serotype 1 group such as an active or inactive (vaccine) rabies virus, in the absence or presence of a mitogenic substance, before hybridisation or, if appropriate, transformation. After hybridisation, the fusion products are screened for those secreting the desired monoclonal antibody.

A preferred strategy, not intended to limit the invention in any manner, for preparing and identifying hybrids that produce antibodies of the invention is as follows. A human volunteer that has been previously immunized against rabies virus is boosted with a commercially available preparation of rabies virus vaccine (Rasilvax (Trade Mark), Sclavo, Italy) consisting of an inactivated and lyophilized suspension of rabies virus (strain Pitman-Moore/Wistar PM/W 381503-3M). After four days, PBL obtained from this donor are separated into a B-cell enriched and a B-cell depleted fraction through panning on goat anti-human immunoglobulin-coated plastic dishes. The cells of the adherent, B-cell enriched fraction, are transformed with EBV and cultured at 1000 cells per microtiter well using irradiated (3000 Rad) mouse peritoneal cells as feeder cells.

Supernatants from the resulting lymphoblastoid cell lines are screened after approximately 15 days in an ELISA for immunoglobulin production and then in an ELISA against Rasilvax for the detection of antibodies binding to determinants defined by the vaccine. Supernatants that score positive in both assays and which exhibit the highest activities in the latter assay are pooled, expanded, and finally fused to HPRT-deficient and ouabain-resistant FD5 cells. Hybrids are selected in a tissue culture medium containing aminopterin and ouabain.

Supernatants are screened in a Rasilvax-ELISA as described in the Example below. Those exhibiting the highest activities in this assay are treated for their ability to neutralise infectious rabies virus.

Cells from wells producing the desired antibodies are cloned by limiting dilution in wells containing irradiated mouse peritoneal cells as feeder cells. The progeny of clones producing the desired antibodies are grown in vitro in suitable culture media in tissue culture flasks or in a hollow fiber tissue culture device (e.g. Acusyst-Jr., Endotronics, Coon Rapids, Minnesota) or in vivo in immunodeficient laboratory animals.

If desired, the antibody may be separated from the culture medium or body fluid, as the case may be, by techniques such as ammonium sulfate precipitation, ion exchange chromotography, affinity chromatography, high-performance liquid chromatography or by other techniques known to those of ordinary skill in the art. According to the preferred strategy, therefore, it is possible to produce human monoclonal antibodies which are characterised in that:

a) their population is substantially homogeneous;

b) they are produced by immortal cells which are themselves hybrids between an immortal cell line and an

antibody-producing human cell;

c) they bind to determinants defined by a rabies virus vaccine; and

d) they neutralise infectious rabies virus so that it can no longer infect target cells.

The monoclonal antibodies of the invention find therapeutic application as part of a suitable post-exposure protocol in individuals at risk to develop rabies. They can be instilled or infiltrated around the wound or administered parenterally together with a vaccine.

The monoclonal antibodies of this invention may be formulated in pharmaceutical compositions by including appropriate amounts of the monoclonal antibody together with a pharmaceutically acceptable carrier or diluent. Generally speaking, these carriers and diluents include or alcoholic/aqueous solutions, emulsions, or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives can also be present such as, for example, antimicrobials, antioxidants, chelating agents, inert gases, and the like. See, generally, Remington's Pharmaceutical Sciences, 16th Ed., Mack, eds., 1980.

Administration of the monoclonal antibody is preferentially by methods presently employed with rabies immune globulin of either human or heterologous origin. Thus, the monoclonal antibody may be instilled or infiltrated around the wound of individuals bitten by aminals and at risk of developing rabies. Moreover it may be administered intramuscularly in a single dose. These treatments can be followed by a complete course of vaccine, see WHO Technical Report Series.

The monoclonal antibody is typically used at doses equivalent to those currently employed with rabies immune globulin of human origin, see WHO Technical Report Series. Monoclonal antibody of the invention may therefore be administered at doses of 0.4-1.6 mg/kg body weight which corresponds to 20-40 International Units (I.U.) of rabies immune globulin of human origin.

The monoclonal antibodies of the present invention are particularly suited for use in immunoassays wherein they may be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibody in these immunoassays can be detectably labeled in various ways.

There are many carriers to which the monoclonal antibody of the present invention can be bound and which can be used in detecting the presence of the rabies virus. Well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agoroses and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody, or will be able to ascertain the same by the use of routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include, but are not limited to, enzymes, radioisotopes, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds and metal chelates. Those of ordinary skill in the art will know of other suitable labels for binding to the monoclonal antibody, or will be able to ascertain the same by the use of routine experimentation. Furthermore, the binding of these labels to the monoclonal antibody can be accomplished using standard techniques commonly known to those of ordinary skill in the art.

One of the ways in which a monoclonal antibody of the present invention can be detectably labeled is by linking the same to an enzyme. This enzyme, in turn, when later exposed to its substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected as, for example, by spectrophotometric or fluorometric means. Examples of enzymes which can be used to detectably label the monoclonal antibodies of the present invention include malate dehydrogenase, staphyloccocal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase.

The monoclonal antibody of the present invention can also be labeled with a radioactive isotope which can then be determined by such means as the use of a gamma counter or a scintillation counter. Isotopes which are particularly useful for the purpose of the present invention are: ^{3}H, ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{36}Cl, ^{57}Co, ^{58}Co, ^{59}Fe, and ^{75}Se.

It is also possible to label the monoclonal antibody with a fluorescent compound. When the fluorescently labeled monoclonal antibody is exposed to light of the proper wave length, its presence can then be detected due to the fluorescence of the dye. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, ophthaldehyde and fluorescamine.

The monoclonal antibody of the invention can also be detectably labeled using fluorescent emitting metals such as ^{152}Eu, or others of the lanthanide series. These

metals can be attached to the antibody molecule using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The monoclonal antibody of the present invention also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged monoclonal antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the monoclonal antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent monoclonal antibody is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Another technique which may also result in greater sensitivity when used in conjunction with the present invention consists of coupling the monoclonal antibody of the present invention to low molecular weight haptens. The haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin (reacting with avidin) or dinitrophenyl, pyridoxal and fluorescamine (reacting with specific anti-hapten antibodies) in this manner.

For the purposes of the present invention, the rabies virus which is detected by the monoclonal antibody of the invention may be present in biological fluids or tissues. Any sample, obtained from an infected animal or human, containing the detectable yet unknown amount of the

virus can be used. Normally, the sample is a liquid, such as, for example, the saliva of an infected animal, cerebrospinal fluid, blood, serum, urine and the like, or a solid or semi-solid, such as, for example, tissue, feces and the like.

The monoclonal antibodies of the present invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therewith one or more container means such as vials, tubes and the like, each of said container means comprising the separate elements of the immunoassay to be used. A similar kit may be prepared comprising compartmentalized carrier means having one or more container means comprising separate elements suitable for therapeutic use according to the present invention.

The types of immunoassays which can be used or incorporated in kit form are many. Typical examples of some of the immunoassays which can utilize the antibodies of the invention are competitive assays and immunometric, or sandwich, immunoassays.

By the term "immunometric assay" or "sandwich immunoassay", it is meant to include simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those of ordinary skill in the art. Those of ordinary skill in the art will also appreciate that the monoclonal antibody of the present invention may be useful in other variations and forms of immunoassays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

In a forward sandwich immunoassay, a sample is first incubated with a solid phase immunoabsorbent containing monoclonal antibody(ies) against the rabies antigen. Incubation is continued for a period of time

sufficient to allow the antigen in the sample to bind to the immobilized antibody in the solid phase. After the first incubation, the solid phase immunoabsorbent is separated from the incubation mixture and washed to remove excess antigen and other interfering substances, such as non-specific binding proteins, which also may be present in the sample. Solid phase immunoabsorbent containing rabies antigen bound to the immobilized antibody is subsequently incubated for a second time with soluble labelled antibody or antibodies. After the second incubation, another wash is performed to remove unbound labelled antibody(ies) from the solid phase immunoabsorbent and removing non-specifically bound labelled antibody(ies). Labelled antibody(ies) bound to solid phase immunoabsorbent is then detected and the amount of labelled antibody detected serves as a direct measure of the amount of antigen present in the original sample. Alternatively, labelled antibody which is not associated with the immunoabsorbent complex can also be detected, in which case the measure is in inverse proportion to the amount of antigen present in the sample. Forward sandwich assays are described, for example, in United States Patents 3,867,517; 4,012,294 and 4,376,110.

In carrying out forward immunometric assays, the process comprises, in more detail:

(a) first forming a mixture of the sample with the solid phase bound antibody(ies) and incubating the mixture for a time and under conditions sufficient to allow antigen in the sample to bind to the solid phase bound antibody(ies).

(b) adding to the mixture after said incubation of step (a) the detectably labelled antibody or antibodies and incubating the new resulting mixture for a time and under

conditions sufficient to allow the labelled antibody to bind to the solid phase immunoabsorbent;

(c) separating the solid phase immunoabsorbent from the mixture after the incubation in step (b); and

(d) detecting either the labelled antibody or antibodies bound to the solid phase immunoabsorbent or detecting the antibody not associated therewith.

In a reverse sandwich assay, the sample is initially incubated with labelled antibody(ies), after which the solid phase immunoabsorbent containing multiple immobilized antibodies is added thereto, and a second incubation is carried out. The initial washing step of a forward sandwich assay is not required, although a wash is performed after the second incubation. Reverse sandwich assays have been described, for example, in United States Patents 4,098,876 and 4,376,110.

In carrying out reverse immunometric assays, the process comprises, in more detail:

(a) first forming a mixture of the sample with the soluble detectably labelled antibody for a time and under conditions sufficient to allow antigen in the sample to bind to the labelled antibody;

(b) adding to the mixture after the incubation of step (a) the solid phase bound antibodies and incubating the new resulting mixture for a time and under conditions sufficient to allow antigen bound to the labelled antibody to bind to the solid phase antibodies;

(c) separating the solid phase immunoabsorbent from the incubating mixture after the incubation in step (b); and

(d) detecting either the labelled antibody bound to the solid phase immunoabsorbent or detecting the labelled antibody not associated therewith.

In a simultaneous sandwich assay, the sample, the immunoabsorbent having multiple immobilized antibodies thereon and labelled soluble antibody or antibodies are incubated simultaneously in one incubation step. The simultaneous assay requires only a single incubation and has a lack of washing steps. The use of a simultaneous assay is by far the preferred method. This type of assay brings about ease of handling, homogeneity, reproducibility, linearity of the assays and high precision. The sample containing antigen, solid phase immunoabsorbent with immobilized antibodies and labelled soluble antibody or antibodies is incubated under conditions and for a period of time sufficient to allow antigen to bind to the immobilized antibodies and to the soluble antibody(ies). In general, it is desirable to provide incubation conditions sufficient to bind as much antigen as possible, since this maximizes the binding of labelled antibody to the solid phase, thereby increasing the signal. Typical conditions of time and temperature are two hours at 45°C, or twelve hours at 37°C.

Antigen typically binds to labelled antibody more rapidly than to immobilized antibody, since the former is in solution whereas the latter is bound to the solid phase support. Because of this, labelled antibody may be employed in a lower concentration than immobilized antibody, and it is also preferable to employ a high specific activity for the labelled antibody. For example, labelled antibody might be employed at a concentration of about 1-50 ng/per assay, whereas immobilized antibody might have a concentration of 10-500 ng/per assay per antibody. Where radiolabelled, the antibody might have a specific

activity with, for instance, one radioiodine per molecule, or as high as two or more radioiodines per molecule of antibody.

In carrying out the simultaneous immunometric assay on a sample containing a multivalent antigen, the process comprises, in more detail:

(a) simultaneously forming a mixture comprising the sample, together with the solid phase bound antibody and the soluble labelled antibody or antibodies;

(b) incubating the mixture formed in step (a) for a time and under conditions sufficient to allow antigen in the sample to bind to both immobilized and labelled antibodies;

(c) separating the solid phase immunoabsorbent from the incubation mixture after the incubation; and

(d) detecting either labelled antibody bound to the solid phase immunoabsorbent or detecting labelled antibody not associated therewith.

Of course, the specific concentrations of labelled and immobilized antibodies, the temperature and time of incubation as well as other assay conditions can be varied, depending on various factors including the concentration of antigen in the sample, the nature of the sample, and the like. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Ater the incubation, the solid phase immunoabsorbent is removed from the incubation mixture. This can be accomplished by any of the known separation techniques, such as sedimentation and centrifugation. Detection can be performed by a scintillation counter, for example, if the label is a radioactive gamma-emitter, or by

a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods which employ a substrate for the enzyme.

Other such steps as washing, stirring, shaking, filtering and the like may of course be added to the assays, as is the custom or necessity for any particular situation.

There are many solid phase immunoabsorbents which have been employed and which can be used in the present invention. Well known immunoabsorbents include beads formed from glass, polystyrene, polypropylene, dextran, nylon and other materials; tubes formed from or coated with such materials and the like. The immobilized antibodies can be either covalently or physically bound to the solid phase immunoabsorbent, by techniques such as covalent bonding via an amide or ester linkages, or by absorption. Those skilled in the art will know many other suitable solid phase immunoabsorbents and methods for immobilizing antibodies thereon, or will be able to ascertain such, using no more than routine experimentation.

The various aspects of the invention are further described by the following example. This example is not intended to limit the invention in any manner.

Example

A. Lymphocyte donor

A vaccinated human volunteer, known to be a high responder for antibody production against rabies virus was booster-immunized with Rasilvax. Fifty ml of venous blood were drawn from the cubital vein after four days.

B. Isolation of human B lymphocytes and transformation with EBV.

The heparinized blood was diluted 1:1 with Ca^{++}-Mg^{++} free Earle's Balanced Salt Solution (EBSS) and then gradient

centrifuged for 40 minutes at 300 x g using commercially available Ficoll/Hypaque (d = 1.077, Lymphoprep, Immuno, Pisa, Italy). Buffy coat lymphocytes were suspended (3 x 10^6 cells/ml) in complete RPMI medium (RPMI 1640 (Flow, McLean, Virginia) supplemented with 20% fetal calf serum (Flow, heat inactivated at 56°C for 30 minutes), 1 mM MEM sodium pyruvate (Gibco, Paisley, Scotland), MEM nonessential amino acids (Gibco), 100 ug/ml of penicillin, 100 ug/ml of streptomycin, 1.5 ug/ml of amphotericin B and additional glutamine (2mM)) and incubated (5 ml/dish) at 4°C for 70 min in a plastic Petri dish (100 mm plastic dishes, Falcon 1005, Becton Dickinson, Mountain View, California) that had been previously coated with goat antihuman immunoglobulin antiserum (Cappel, Malvern, Pennsylvania, 20 ug/ml in 20 mM phosphate buffer, 5 ml/dish).

At the end of the incubation period, supernatant medium containing nonadherent cells was aspirated and adherent cells were detached through vigorous pipetting. Nonadherent cells were shown to be B-cell depleted and adherent cells B-cell enriched as judged by immunofluorescence studies with anti-human immunoglobulin antibodies, which detect immunoglobulins on the surface of B-lymphocytes. The B cell-enriched fraction was transformed with EBV by incubation for two hours at 37°C with a supernatant of the EBV producing marmoset cell line B95-8 (Miller, G., et al., Proc. Natl. Acad. Sci. USA 69:383-387 (1972)).

At the end of the treatment, the cells were washed, resuspended in complete RPMI medium and cultured at 10^3 cells/well in 96-well tissue culture plates (Falcon No. 3072) at 37°C, under 5% CO_2. The culture plates were assayed after 15 days by an Ig ELISA and Rasilvax ELISA (described below). Antibody cultures that were strongly positive in this latter assay were pooled and expanded in tissue culture falsks (Costar No. 3050, Cambridge,

Massachusetts) until a sufficient number of cells for the fusion process was available. Table 1 shows the results of these assays.

C. Fusion protocol

Fusion partners were cells from the pool of EBV-transformed cell cultures that had been obtained as described in the preceding section and cells from the HPRT-deficient and ouabain-resistant FD5 cell line. This latter cell line secretes negligible amounts of IgG. The fusion mixture contained polyethylene glycol (PEG) 1500 (Serva, Heidelberg, West Germany) 40% w/v in complete RPMI medium without fetal calf serum.

Parent cells were washed twice in serum-free complete RPMI medium at room temperature and subsequently resuspended and combined at a ratio of 1:1 of EBV-transformed cells: FD5 cells in serum-free RPMI warmed at 37°C in a conical 50 ml centrifuge tube (Falcon No. 2070).

Upon centrifugation for 10 minutes at 400 g, the medium was discarded and 2 ml of the PEG fusion mixture was carefully added drop by drop over a 1 minute period. Thereafter, the mixture was diluted slowly with serum-free complete RPMI medium. The volume was adjusted to give a cell density of 5×10^5 FD5 cells/ml. 200 ul of this suspension was seeded into each well of 96-well plates that had been previously seeded with irradiated mouse peritoneal cells (5×10^4 cells/well). Plates containing the fusion products were then incubated for 24 hours at 37°C, 5% CO_2.

The culture medium was then replaced with complete RPMI medium containing hypoxanthine (10^{-4}M), aminopterin (4×10^{-7}M), thymidine (1.6×10^{-5}M) and ouabain (10^{-6}M). This medium is hereafter referred to as HAT-O medium. In this culture medium, only hybridomas can survive, since FD5 cells are killed in the presence of aminopterin and EBV-transformed cells are killed in the presence of ouabain.

The plates were then further incubated at 37°C, 5%

CO_2 for 5-6 weeks. During this period, the cultures were fed every three days with fresh medium. Growing hybrids were visible by day 15. Ouabain was removed from the medium at day 10 of this period, aminopterin at day 30, and complete RPMI medium was used starting from the sixth week.

At the end of the time period indicated above, cultures were tested for production of antibodies binding to determinants defined by a rabies virus vaccine by Rasilvax-ELISA and finally for the production of virus-neutralizing antibodies as measured by a rapid fluorescent focus inhibition test (RFFIT; for a detailed description of the assays, see below). The results of these assays are reported in Table 2 and Figure 1. Hybrid cells producing the supernatant 208 that determined the strongest inhibition in the virus-neutralizing antibody assay were cloned by limiting dilution at an input of 0.5 cells/well. After three weeks the percentage of growth-positive wells was determined. Growth-positive wells were tested for Ig production and production of antibodies binding to determinants defined by a rabies virus vaccine as measured by Rasilvax-ELISA. The results of these assays are summarised in Table 3.

Supernatants showing the strongest antigen binding activity were tested for their capacity to neutralize infectious rabies virus as measured by a RFFIT. The results are shown in Figure 2. In this set of experiments supernatants (1:10 final dilution) were incubated for 1 h at 37°C with 10^5 (panel a), 10^3 (panel b) or 10^2 (panel c) infectious rabies virus particles. As can be seen, under conditions of lower antigen input complete or almost complete neutralization could be observed.

Clone 208/97 was further expanded for a more detailed characterization of its properties. Table 4 summarizes some of the data that have been obtained. It is noteworthy that the monoclonal antibodies produced by this clone neutralize

two different strains of rabies virus (CVS and ERA) thus showing that they recognize a common epitope.

Immunofluorescence studies (Fig. 3) showed that monoclonal antibodies produced by clone 208/97 stained only infected cells. They bound to both fresh (membrane fluorescence) as well as fixed cells. This shows that the monoclonal antibodies recognized viral determinants expressed on the membrane, as expected for a neutralizing antibody recognizing the G protein of rabies virus (Cox, J.H., et al, Infect. Immun. 16: 743 (1977)). On the other hand a polyclonal antiserum against viral nucleocapsid, an antigen expressed only in the cytoplasm of infected cells, bound only to infected, fixed cells (cytoplasmic fluorescence).

The neutralizing potency of the antibodies produced by clone 208/97 was compared with that of a reference equine antirabies serum. The results are shown in Figure 4. As can be seen, 10 µg/ml of the monoclonal antibody give an inhibition equivalent to that obtained with 0.5 I.U. of the polyclonal antibody preparation.

In a further experiment 10^3 infectious virus particles were allowed to adsorb for 1 hour on to target chicken-embryo related (CER) cells under conditions (4°C) that precluded virus internaliziation. At the end of the incubation period cells were washed free of unadsorbed virus and fresh culture medium with or without monoclonal antibody 208/97 or polyclonal antiserum was added to the cells.

The cells were then incubated for 1 h at 37°C. At this point the antibody-containing medium was replaced with fresh culture medium and the cells were further cultured under standard conditions. The results are shown in Figure 5. As can be seen the antiserum was not protective under these conditions. The monoclonal antibody, however, still showed a protective effect. These results suggest that the monoclonal antibody of the present invention may be even

more effective than an antiserum because it has the capacity to partially protect target cells from infection through adsorbed viruses, whereas a polyclonal antiserum is incapable to do so.

Hybridoma 208/97 has been deposited with the ECACC under accession number 88021901 on 19 February 1988.

D. Methods

1. Immunoglobulin-ELISA

Flat-bottom microtiter plates (Falcon No. 3912) were coated at 100 µl/well with goat anti-human immunoglobulin (Ig) antibody (20 µg/well, Cappel) diluted in 20 mM phosphate buffer (pH 9.6). After an overnight incubation at 4°C, plates were washed two times with Washing Buffer (WB) pH 8, containing Tween-20 0.05% and 10 mM Tris (hydroxy-methylaminomethane) (NEN, Boston, Massachusetts).

Phosphate-buffered saline (PBS) with 1% BSA (Armour Pharmaceutical Co., Kanakee, Illinois) was then added to each well to saturate unoccupied plastic sites. The plates were then further incubated for 30 minutes at room temperature. Thereafter, plates were washed as above. Test supernatants (100 ul) were added to each well, plates incubated for 1 hour at 37°C and then washed as above. Then, 100 ul of peroxidase-conjugated $F(ab')_2$ fragment goat anti-human Ig (IgA + IgG + IgM; Cappel) or rabbit anti-human IgM (µ-chain specific; Dakopatts, Copenhagen, Denmark) properly diluted in PBS (pH 7.4, containing Tween 20, 0.05% (NEN)), were added to each well and the plates further incubated for 60 minutes at 37°C as above.

Then, 200 ul of peroxidase substrate (NEN) was added to each well. This substrate consists of distilled water containing 0.2% of o-phenylenediamine (OPD) and 0.015% of H_2O_2. The reaction was allowed to proceed for 5-15 minutes, after which color development was stopped by the addition of 4M H_2SO_4. The extent of color development within the wells was read on a plate ELISA reader at 492 nm.

For quantitation of Ig levels a human Ig standard (Miles Scientific, Naperville, Illinois) was added to calibrate the system.

2. Rasilvax-ELISA

Flat bottom microtiter plates were coated at 100 ul/well with 50 ug/ml of Rasilvax diluted in 50 mM bicarbonate buffer, pH 9.6. After an overnight incubation at 4°C, the plates were washed twice with WB. PBS with 1% BSA was then added to each well to saturate unoccupied plastic sites and the plates further incubated for 30 minutes at room temperature. Thereafter, plates were washed as above. Test supernatants (100 ul) were added to each well, and the plates incubated for 60 minutes at 37°C, then washed as above. Then, 100 μl of peroxidase-conjugated goat anti-human total Ig (Cappel) diluted 1:600 in PBS supplemented with Tween 20 (0.05%) was added to each well and the plates further incubated for 60 minutes at 37°C as above.

Peroxidase substrate addition and color development was as for Ig-ELISA.

3. Immunofluorescence IF studies

Binding of monoclonal antibodies 208/97 to rabies-virus infected or uninfected cells was studied with indirect immunofluorescence. CER cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 5% newborn calf serum (NCS) in eight chamber microtissue chamber/slides (Miles) for 24 h at 37°C, 5% CO_2. Cells were then washed 3 times with serum-free EMEM and then infected with CVS (challenge virus strain) fixed rabies virus diluted in EMEM 1% NCS for 1 h at 37°C. The inocula were then removed, cells washed with serum-free EMEM and further incubated for a time period sufficient to have 100% of the cells infected. Cells were then washed 3 times with PBS and used either without any fixation or fixed in acetone for 5' at -20°C. In direct IF studies cells were then covered with

fluorescein isothiocyanate (FITC) conjugated rabbit anti-rabies virus nucleocapsid IgG (Institut Pasteur Production, Paris, France) diluted 1:40 in PBS and incubated for 45' at 37°C (fixed cells) or at 4°C (fresh cells). In indirect IF studies cells were first incubated for 45' at 37°C (fixed cells) or at 4°C (fresh cells) with PBS 2% fetal calf serum (FCS) containing 2 µg/ml of monoclonal antibody 208/97. Cells were then washed and further incubated as above, at 37°C or 4°C, with a 1:10 dilution of FITC-conjugated rabbit anti-human Ig (Boehringer, Mannheim, FRG).

Finally, cells were washed with PBS, mounted in glycerol and observed under a fluorescence microscope.

4. Virus neutralization

Virus-neutralizing antibodies were measured by a direct immunofluorescene technique (RFFIT). The neutralizing effect of hybridoma supernatants was determined by incubating 200µl of the indicated dilution of hybridoma or control supernatant with an equal volume of EMEM 1% FCS containing different numbers of infectious rabies virus particles for 1 hour at 37°C.

Rabies virus was CVS or ERA strain of SAD virus propagated in BHK-21 cells. At the end of the incubation period each supernatant mixture was added to CER cells grown in microtissue chamber/slides. After 1h at 37°C, the inocula were removed and the monolayers were washed with EMEM. Cells were further incubated at 37°C, 5% CO_2 for a time period sufficient to have 100% of the cells in control cultures (virus alone) infected. The percentage of infected cells was then determined in direct IF on fixed cells using FITC-conjugated rabbit anti-rabies virus nucleocapsid IgG as described in section 3. Results are expressed as per cent fluorescence inhibition in cell cultures treated with antibody/virus mixtures versus cells treated with virus alone.

In one set of experiments we studied neutralization

after virus adsorption. In this case 10^3 infectious CVS rabies virus particles were allowed to adsorb to CER cells grown in microtissue chamber/slides by incubation for 1 h at 4°C. Cells were then washed with serum-free EMEM and incubated for 1 h at 37°C with either prewarmed EMEM 1% FCS alone or EMEM 1% FCS containing monoclonal antibodies 208/97 or an international reference anti rabies serum. Cells were then washed as above and further incubated for 72 h at 37°C with EMEM 5% NCS. The number of infected cells was then determined as previously described.

5. Chromosome preparation

Chromosome preparations of the 208/97 hybridoma cells were made by use of the air drying technique described by Ishihara et al., Cancer Res., 22:375-379, (1962).

TABLE 1 - EPSTEIN-BARR VIRUS TRANSFORMATION OF B-LYMPHOCYTES ISOLATED FROM THE PERIPHERAL BLOOD OF A DONOR IMMUNIZED WITH A RABIES VIRUS VACCINE.

N° of wells seeded	Wells positive for growth	Wells positive for Ig production	Wells positive for antigen binding[a]	
			Weakly positive (O.D. $>$ 0.4 and $<$ 0.8)	Strongly positive (O.D. $>$ 0.8)
600	600	600	66	42

[a] Determined by Rasilvax-ELISA. The optical density (O.D.) background (determined in the absence of the first antibody) ranged typically between 0.2 and 0.3.

TABLE 2 - HUMAN/HUMAN HYBRIDOMAS DERIVED FROM FUSIONS BETWEEN EBV-TRANSFORMED CELL LINES PRODUCING ANTI-RABIES VIRUS ANTIBODIES AND THE HUMAN CELL LINE FD5.

N° of wells seeded	Wells positive for growth	Wells positive for antigen binding[a]	
		Weakly positive (O.D. > 0.4 and < 0.8)	Strongly positive (O.D. > 0.8)
650	318	74	111

[a] See Table 1

TABLE 3 - CLONES DERIVED FROM HYBRIDOMA 208

N° of wells seeded (0.5 cells/well)	Wells positive for growth	Wells positive for IgM production	Wells positive for antigen binding[a]	
			Weakly positive (O.D. > 0.4 and < 0.8)	Strongly positive (O.D. > 0.8)
600	173	103	70	33

[a] See Table 1

TABLE 4 - PRODUCTION OF HUMAN MAbs BY HUMAN x HUMAN HYBRIDOMA 208/97

Trait	
Growth rate (doubling time)	36 h.
Modal chromosome number	tetraploid
MAb production ($\mu g/10^6$ cells/day)	2
Isotype of MAb	IgM
Neutralizing effect(%) at 1:10(final)dilution	
on the CVS strain	90
on the ERA strain	80

CLAIMS

1. A human monoclonal antibody which:

(a) binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae, and

(b) neutralises said viruses.

2. A human monoclonal antibody according to claim 1, wherein the said antibody is of the IgM immunoglobulin class.

3. A human monoclonal antibody according to claim 1, wherein the said antibody is of the IgG immunoglobulin class.

4. A human monoclonal antibody according to claim 1, which binds specifically to the rabies virus strain Pitman-Moore and neutralises specifically the rabies virus strains CVS and ERA.

5. A hybridoma cell line and progeny thereof which secrete a human monoclonal antibody which:

(a) binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae, and

(b) neutralises said viruses.

6. The hybridoma cell line 208/97 (ECACC accession number 88021901) and progeny thereof.

7. A process for the preparation of a hybridoma cell line which secretes a human monoclonal antibody which:

(a) binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae; and

(b) neutralises said viruses;

which process comprises fusing immortalizing cells with cells derived from a human which produce antibody which binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae.

8. A process according to claim 7, wherein the immortalizing cells are cells of the cell line FD5 (ECACC accession number 87061701).

9. A process according to claim 7 wherein the

antibody-producing cells derived from a human are human lymphocytes transformed with a virus.

10. A process according to claim 7, wherein the antibody-producing cells derived from a human are non-transformed human lymphocytes.

11. A process according to claim 9 or 10, wherein the lymphocytes are stimulated in vitro with a virus of the serotype 1 group of the rabies virus group of Rhabdoviridae and/or a mitogenic substance before being fused or, if appropriate, transformed.

12. A process for the preparation of a human monoclonal antibody which:

(a) binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae; and

(b) neutralises said viruses;

which process comprises culturing a hybridoma cell line or progeny thereof which secretes the said antibody and recovering the monoclonal antibody thus-produced.

13. A process according to claim 12 wherein the hybridoma cell line is cell line 208/97 (ECACC accession number 88021901).

14. A pharmaceutical composition comprising a human monoclonal antibody which:

(a) binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae; and

(b) neutralises said viruses;

and a pharmaceutically acceptable carrier or diluent.

15. The cell line FD5 (ECACC accession number 87061701).

16. A method of detecting whether a sample contains a virus of the serotype 1 group of the rabies group of Rhabdoviridae, which method comprises contacting the sample with a human monoclonal antibody which:

(a) binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae; and

(b) neutralises said viruses;

and detecting whether an antibody-antigen complex has formed.

17. A method of treating a human suspected of being at risk of developing rabies, which method comprises administering thereto an effective amount of a human monoclonal antibody which:

(a) binds specifically to viruses of the serotype 1 group of the rabies group of Rhabdoviridae; and

(b) neutralises said viruses.

1/4

Fig.1

DETECTION OF NEUTRALIZING ANTIBODIES IN HYBRIDOMA SUPERNATANTS

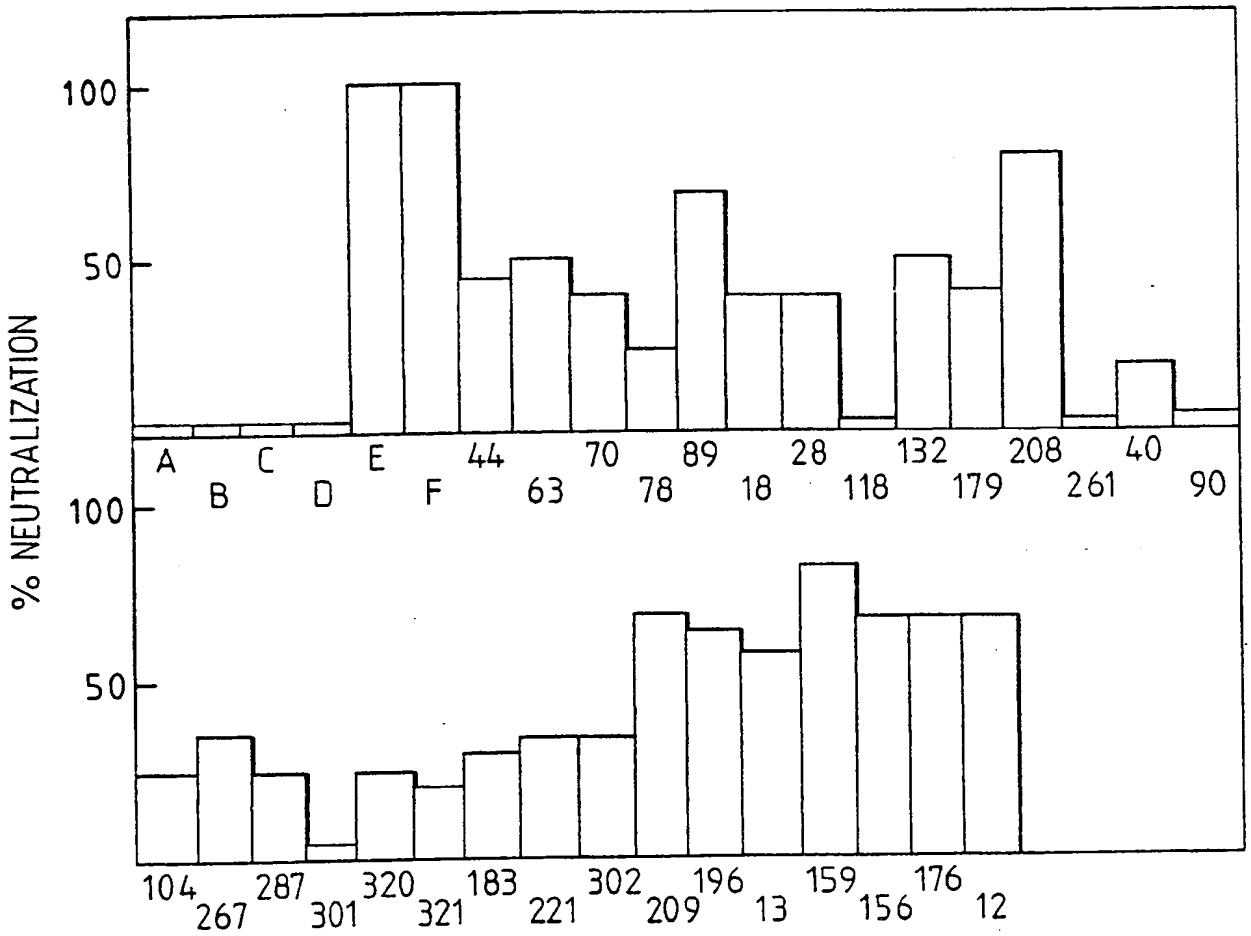
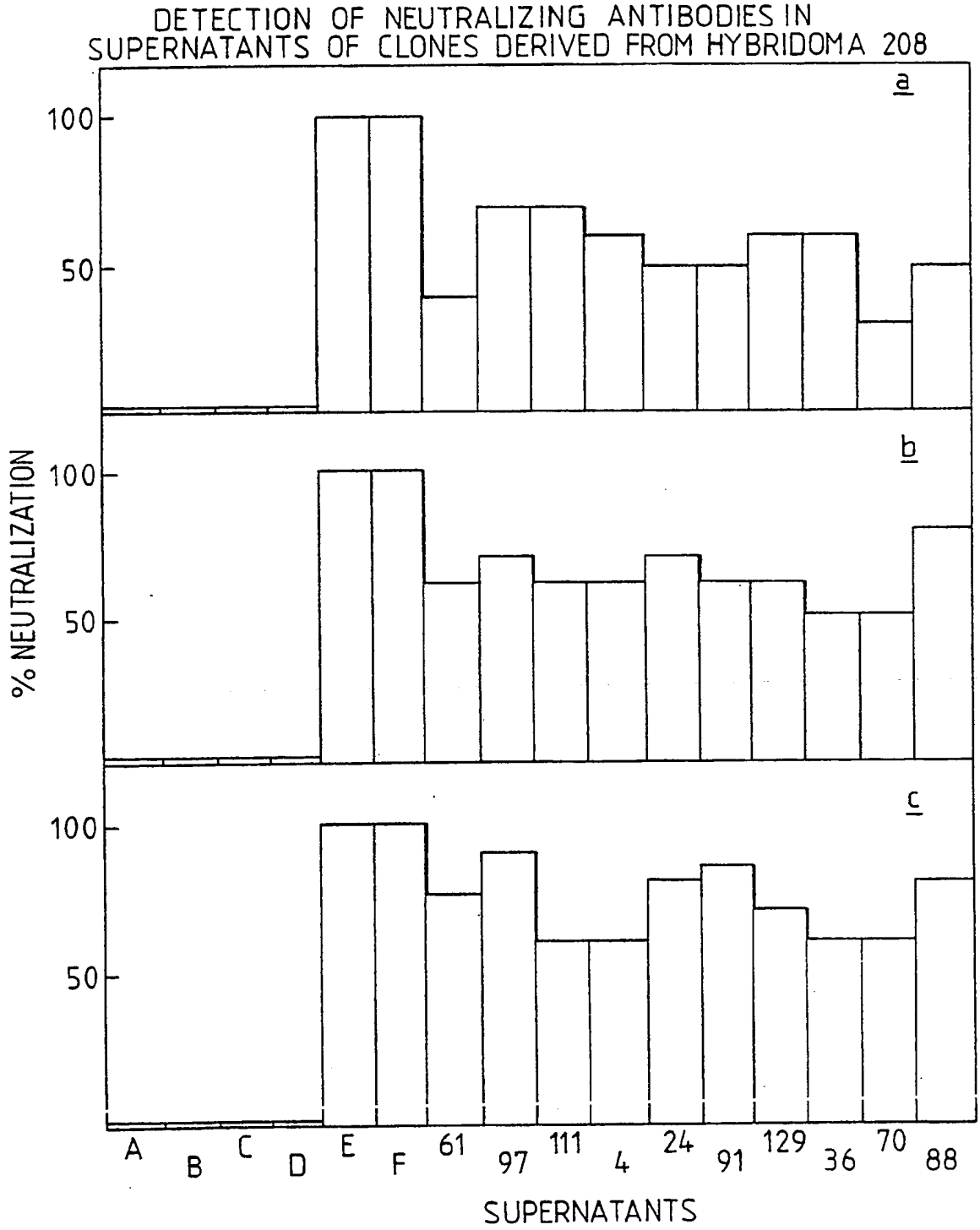


Fig.2


DETECTION OF NEUTRALIZING ANTIBODIES IN SUPERNATANTS OF CLONES DERIVED FROM HYBRIDOMA 208
a
b
c
100
50
% NEUTRALIZATION
A
B
C
D
E
F
61
97
111
4
24
91
129
36
70
88
SUPERNATANTS

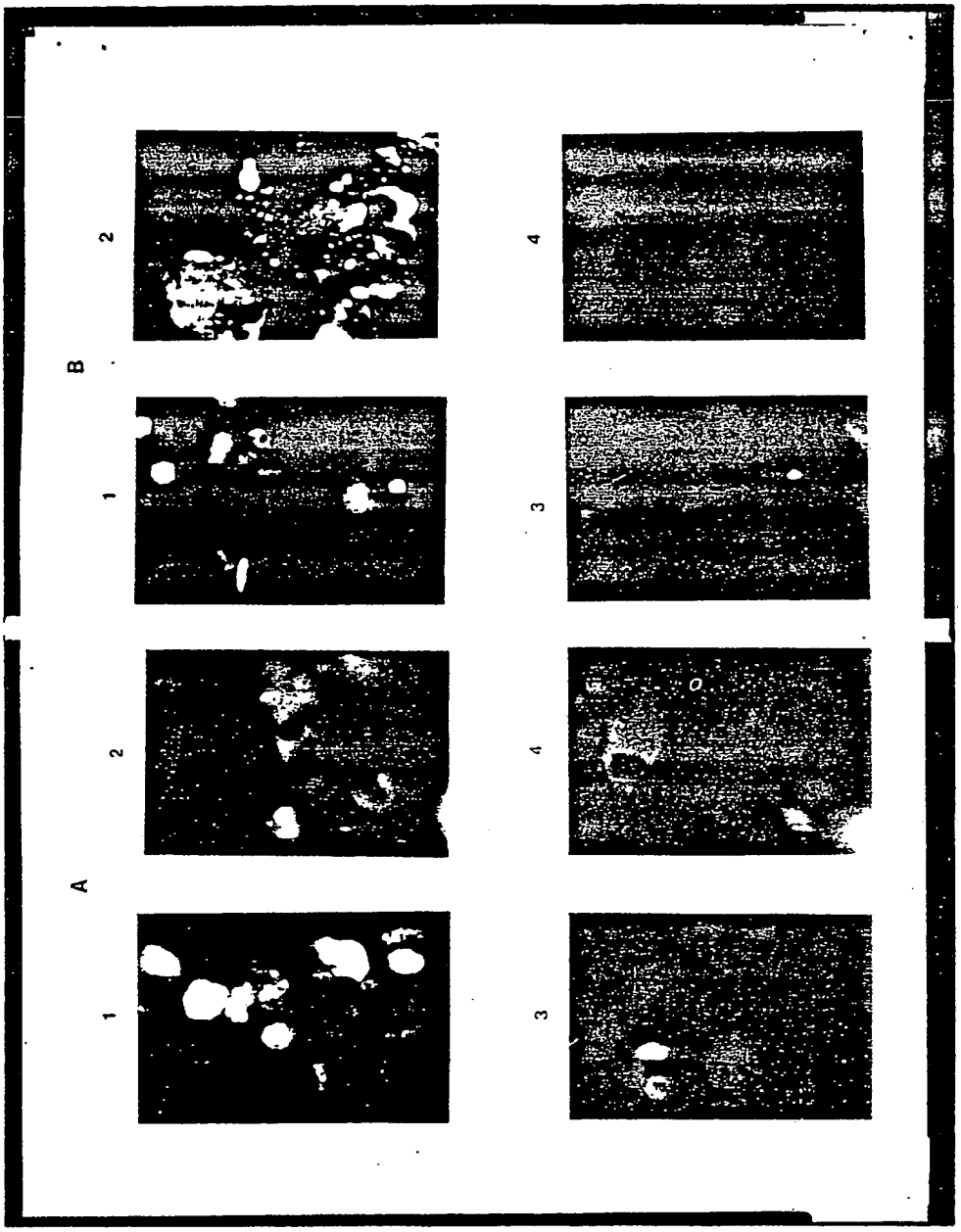


FIG. 3

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 8900365 SA 27757

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 19/07/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA-A- 1212913	21-10-86	None	
WO-A- 8700179	15-01-87	US-A- 4652629	24-03-87
		CA-A- 1248894	17-01-89
		EP-A- 0227824	08-07-87
		JP-T- 63500659	10-03-88
		US-A- 4707356	17-11-87
GB-A- 2086937	19-05-82	AU-B- 548738	02-01-86
		AU-A- 7716781	13-05-82
		BE-A- 891003	01-03-82
		CA-A- 1198066	17-12-85
		CH-B- 658070	15-10-86
		DE-A- 3144181	09-09-82
		FR-A,B 2493865	14-05-82
		JP-A- 57126424	06-08-82
		SE-A- 8106528	08-05-82
		US-A- 4608337	26-08-86